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Enhancement of bacterial denitrification for nitrate removal in groundwater with electrical stimulation from microbial fuel cells

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HIGHLIGHTS

- Electricity from the MFC is applied to the BER directly as electrical stimulation.
- Nitrate removal from groundwater is accelerated by this means.
- Less intermediates accumulation is observed during that process.
- Denitrification bacteria proliferations and activities are promoted.

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ABSTRACT

Electricity generated from the microbial fuel cell (MFC) is applied to the bioelectrical reactor (BER) directly as electrical stimulation means for enhancement of bacterial denitrification to remove nitrate effectively from groundwater. With maximum power density of 502.5 mW m^{-2} and voltage outputs ranging from 500 mV to 700 mV, the nitrate removal is accelerated, with less intermediates accumulation, compared with control sets without electrical stimulation. Denitrification bacteria proliferations and activities are promoted as its number and Adenosine-5'-triphosphate (ATP) concentration increased one order of magnitude (3.5×10^7 in per milliliter biofilm solution) and about 1.5 folds, respectively. Effects of electricity from MFCs on enhancement of bacterial behaviors are demonstrated for the first time. These results indicate that MFCs can be applied in the in-situ bioremediation of nitrate polluted groundwater for efficiency improvement.

1. Introduction

Nitrate pollution in groundwater has become a serious issue in most parts of the world during past decades due to discharge of domestic, industrial wastewater and increased usage of nitrogenous fertilizers [1]. Excessive nitrate can do harm to humans and animals and it can be reverted to nitrite with more toxicity by microorganisms in human body, causing methemoglobinemia or blue baby syndrome in infants and gastrointestinal cancer in adults [2]. Thus the maximum contaminant levels (MCL) are stipulated to be 10 mg L^{-1} nitrate nitrogen (NO_3^-–N) and 1 mg L^{-1} nitrite nitrogen (NO_2^-–N) respectively by both USEPA and China.

Various physical–chemical and biological methods have been developed for nitrate removal, such as ion exchange [3], reverse osmosis [4], catalysis reduction [5] and bacterial denitrification [6]. The former can only separate nitrate from polluted groundwater, and cumbersome treatments are often inevitable, while the latter is able to remove nitrate efficiently and cost-effectively, drawing more and more attentions nowadays [7]. Bacterial denitrifications, both heterotrophic and autotrophic modes, are considered to be employed for nitrate removal [6,8].

To improve efficiencies of bacterial denitrification for nitrate removal, several meanings are employed, including nutrient elements and electron donors adjustment by extra addition (organics

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and phosphorus) or electrochemically in-situ generation (hydrogen) [9]. These may increase operating costs or consume extra electric energy. Moreover, it is believed that proper electrical stimulation can promote microbial metabolism, thereby leading to higher biochemical performance [10]. Direct-current power supplies are used in almost all these previous studies and operated in relatively higher voltage outputs, ie above the decomposition voltage of water (1.2 V vs NHE) to obtain hydrogen [10]. Few studies are carried out to investigate the behaviors of denitrification under lower voltage without water decomposition, as some research reveals that low voltage stimulation (less than 1.5 V) without electrolytic O_2 generation can enhance the microbial degradation of PCB, by providing electron-donors/-acceptors to PCB dechlorination and microorganisms degradation [11]. Nowadays, microbial fuel cells (MFCs), devices that use bacteria as catalysts to oxidize organic or inorganic matters and generate current, are attracting increasing attention as they can convert chemical energy to electricity in mere one step [12–15]. Most reported voltage outputs from MFCs are less than 1 V and have been used to power sensors and generate hydrogen [16], while few studies concern on the enhancement of denitrification bacteria activities by directly electrical stimulation from MFCs for nitrate removal.

In the present research, the in-situ utilization of low voltage generated by MFC was considered. Electricity generated from MFCs was applied to bacterial denitrification directly as electrical stimulation means for effective nitrate removal from groundwater. The ability of voltage outputs of the MFC was evaluated. Nitrate removal and its reduced products in both aqueous solution and gaseous phase were measured. The enhancement of denitrification bacteria proliferations and activities under the stimulation of this low voltage was also monitored.

2. Materials and methods

2.1. Experimental apparatuses and electrolyte

The configuration of the experimental apparatus was shown in Fig. 1. It consisted of an MFC and a bioelectrical reactor (BER), with another two electrochemical cells as controls. The single-chamber MFC was in cubic shape and had been reported in our previous study [17], with an effective volume of 125 mL (5 cm × 5 cm × 5 cm). The anode was carbon fiber felt (1 cm thickness, 4 cm length and width, Beijing Ever grow Resources Co. Ltd, Beijing, China). The cathode made of plain carbon paper (with 0.5 mg cm^{-2} of Pt on one side) with a projected surface area of 16 cm^2 was placed on the opposite site of the anode. The voltage outputs of the MFC were recorded by a data acquisition system (PMD1208LS, Measurement Computing Corp., Norton, MA, USA) at an interval of 5 min [18]. The MFC had been well developed before present experiment. The electrolyte contained the following components (per liter): $C_6H_{12}O_6$ (0.75 g); NH_4Cl (0.31 g); KCl (0.13 g); $NaH_2PO_4 \cdot H_2O$ (4.97 g); $Na_2HPO_4 \cdot H_2O$ (2.75 g); vitamin solution (1.25 mL) and 12.5 mL trace mineral element solution.

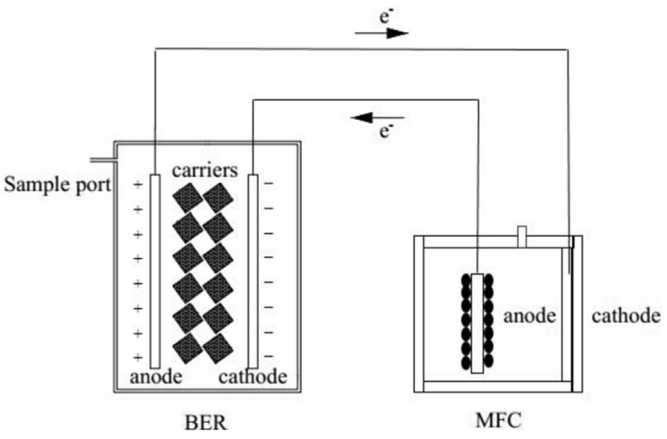

Fig. 1. Experimental apparatus. The left: MFC unit, 1 cathode (Pt coated carbon paper); 2 anode (carbon fiber felt). The right: BER unit, 3 anode (stainless steel plate); 4 cathode (stainless steel plate); 5 carriers (functional polyurethane foams).

The BER was designed in sealed cuboid shape and the total volume was 480 mL. Both anode and cathode were made of stainless steel (15 cm × 4.3 cm), with the electrode spacing of 2 cm. Functional Polyurethane Foams (FPF) with the specific surface area of 35,000 m^2 m^{-3} were added as carriers to immobilize the microorganisms in the BER [17]. The electrodes of MFC were connected to those of BER using copper wires. Another two electrochemical cells were built in the same specification as the BER. One was not inoculated (Control 1) and another was inoculated (Control 2). The BER and the Control 2 were respectively inoculated with 50 mL anaerobic sludge, which was collected from the Qinghe Sewage Treatment Plant (Beijing, China) and had been well acclimated before the formal trial. Synthetic groundwater (per liter of tap water) contained 0.364 g $NaNO_3$, 0.044 g KH_2PO_4, and 0.21 mL CH_3OH. The concentration of NO_3^-–N was prepared as around 60 mg L^{-1}.

2.2. Experimental procedure

The MFC and BER as well as control sets were filled with fresh electrolyte and synthetic groundwater, respectively. The BER and Control 2 were domesticated separately for 4 weeks to achieve the same performance before the formal experiments. Then BER was connected with MFC through copper wires to domesticate denitrification bacteria in the BER to accommodate the electrical stimulation for about 30 days, with refreshing electrolyte and synthetic groundwater every 3 d and 5 h, respectively. After that, Nitrate removal in a typical cycle was evaluated compared with the control sets. Intermediates of nitrate reduction, both in aqueous solution and gaseous phase, were also measured at the same frequency. Differences of denitrification bacteria in the aspects of amount and activity between BER and Control 2 were investigated to reveal the mechanisms of electrical stimulation, compared with electrolysis effect simulated in Control 1. The abilities of electricity generation in MFC were evaluated during these procedures as well. All the experiments were carried out at room temperature (25 ± 2 °C).

2.3. Analytical methods

NO_3^-–N, NO_2^-–N and NH_4^+–N in the aqueous solution of BER were determined by ultraviolet spectrophotometer (DR 5000, HACH, the USA) according to standard methods, with three duplications for each data point. Chemical oxygen demand (COD) was measured by the standard method of potassium dichromate. N_2O and N_2 in the gaseous phase of BER was detected by gas chromatography (GC, 7890A, Agilent, the USA) with electrical conductivity detector (ECD) and mass spectrometry (MS), repectively [19]. N_2O in the liquid phase was estimated using Henry's function. NO was monitored by nitrogen oxides gas analyzer (42i series, Thermo Fisher, the USA) [20].

Adenosine-5'-triphosphate (ATP) reflecting the activity of denitrification bacteria was quantitatively evaluated by enzymes labeling instrument (Zenyth 340rt, Biochrom Anthos, Austria) with CellTiter-Glo luminescent cell viability assay, which was performed according to previous research and luminescences in the form of RLU (relative light unit) were recorded, as they have a linear

correlation in the range of 890–160,000 RLU [21]. The population densities of denitrification bacteria were determined by the most probable number (MPN) technique, which was applied to quantify nitrate reducers in soil [22]. The biofilm solution for MPN count was extracted from the BER at 2.5 h and taken from the surface of FPF in the middle part of the BER. The sampling point was located at 68 mm from the bottom. Then sludge samples were with control of growth medium. A 10-fold dilution series of samples was prepared using deionized water, and 10 mL of each dilution was added into 100 ml of the sterilized growth medium in 250 mL conical flasks (four replicates per dilution stage). The growth medium (per liter of deionized water) contained 2.0 g KNO_3, 5.0 g $C_6H_5Na_3O_7$, 0.2 g $MgSO_4 \cdot 7H_2O$, 1.0 g K_2HPO_4, 1.0 g KH_2PO_4 and the pH was adjusted to 7.2 by 1 mol L^{-1} HCl or NaOH solution. It was sterilized at 121 °C for 30 min in autoclave before use. The flasks were aerated by nitrogen to avoid oxygen, and then incubated in a constant temperature horizontal shaking bath container (30 °C, 160 revolutions per minute; DDHZ-300, Taichang, China) for 14 days. The number of positive wells for each dilution was calculated as described by Briones and Reichardt [23]. As the comparison, 10 mL deionized water was added into 100 mL of the sterilized growth medium in 250 mL conical flasks (4 replicates).

2.4. Electrochemical monitoring and data presentations

Anodic and cathodic half-cell potentials of the BER were measured by placing Ag/AgCl reference electrode in the BER. Polarization curves of the MFC were obtained to evaluate the relationship between voltage and current by measuring voltages at various external resistances (ranging from 10 to 5000 Ω). For each point on the polarization curves, voltage readings were taken by a multimeter when the voltage stabilized. When the MFC was worked as a power supply unit, the voltage and current were monitored via the data acquisition system continuously. Power density (PD, W m^{-2}) was calculated according to $PD = I \times U/S$, where S (m^2) is the geometrical area of the anode, U and I are voltage (V) and current (A), respectively.

3. Results and discussion

3.1. Power outputs of the MFC and evaluation of its power supply ability

The MFC had been steadily operated for half a year with 750 mg L^{-1} glucose before the present study. The effective microbial biofilm was well developed on the anode surface. When 1000 Ω external resistance was connected, the voltage outputs ranged from 200 mV to 700 mV during a 72 h operation. Polarization curve was performed with closed circuit and maximum power density of 502.5 mW m^{-2} was obtained by varying external resistances (Fig. 2), which was comparable with single-chamber MFC systems utilizing simple organic substrate such as glucose or acetate reported previously [24]. This demonstrated that this organics could be effectively utilized by microbes for power production and the MFC had the ability to output electricity to power electrical appliances.

When the BER was connected to the MFC directly, replacing the fixed external resistance, the voltage outputs monitored during 10 d operation with fresh anolyte were 100–700 mV, which were much lower than voltages applied in previous studies for biological nitrate removal [11]. Electricity from the MFC could also be applied to BERs and might also have the function of accelerating nitrate removal. Denitrification under these lower voltages without water decomposition might exhibited different behaviors. Both of which were worth to be investigated.

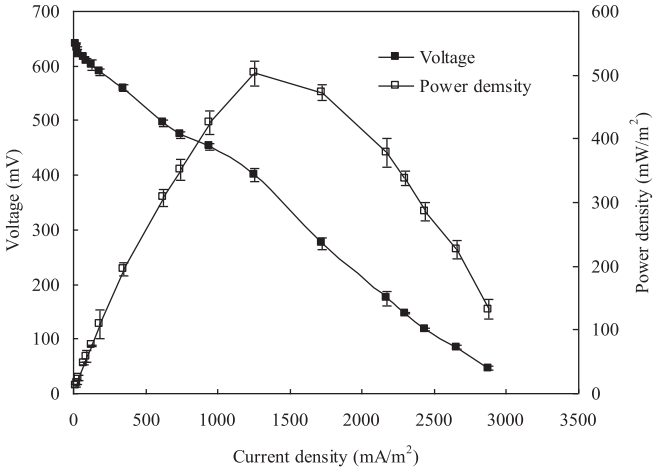

Fig. 2. Polarization curve and power outputs of the single-chamber MFC.

3.2. Nitrate removal and its reduction products identification in the BER

3.2.1. Performance of nitrate removal

After 4 weeks domestication, the BER and Control 2 reached the similar nitrate removal ability, with residual NO_3^-–N of about 3 mg L^{-1} in both effluents after 3 h, though it could be exhaused in the end of the operating cycle. Then the BER was connected to the MFC for electrical acclimatization for 30 d. After that, The BER as well as control 1 and 2 was filled with fresh synthetic groundwater and operated synchronously, with the applied voltages of BER and Control 1 settled to be 500–700 mV, with current outputs of 0.8–1.2 mA NO_3^-–N removal tendencies with time were shown in Fig. 3a, which indicated that nitrate removal was substantially enhanced in BER. In a typical operating cycle (5 h), NO_3^-–N concentration decreased gradually and it was removed completely in the BER (Fig. 3b). Nitrate could also be removed in Control 2 due to denitrification bacteria in the inoculated sludge with relatively slower rate, especially under higher nitrate concentration. The regularity of nitrate removal rate in Control 2 was stable during the process of electrical acclimatization of MFC intensified BER (30 d) and the formal experiments. Nitrate concentration change was rarely observed in Control 1.

Accelerative mechanisms of nitrate removal were distinguished. The applied electric field could enhance the nitrate removal physically, chemically and biologically. Firstly, it had been discovered that nitrate could be reduced electrochemically in an undivided cell using Cu–Zn alloy as cathode and Ti/IrO_2–Pt as anode in our previous studies [25], but the applied voltage (above 7.6 V) was much higher than that from the present MFC, indicating that the electrical promotion of denitrification was not caused by direct electrode oxidation or electrolysis of water to hydrogen as reported before in this research [11]. In fact, based on the assumption that all the electrons from the MFC (about 18 C) could transfer to nitrate, its contribution to the total nitrate removals was only 2.7%, which could be neglected, in other words, the dominant electron source for nitrate reduction was CH_3OH. Electrons from CH_3OH were mainly contributed to both bacteria growth and nitrate removal. Secondly, mass transfers and ion migrations were also examined. Electric mobility of NO_3^- and Na^+ under 298 K in infinite dilute aqueous solution were 7.40×10^{-8} m^2 (s V)$^{-1}$ and 5.20×10^{-8} m^2 (s V)$^{-1}$, respectively. With electricity from the MFC applied, the values as calculated as Eq. (1) were 5.36×10^{-8} m^2 (s V)$^{-1}$ and 3.68×10^{-8} m^2 (s V)$^{-1}$, in turn, suggesting ion migrations were hardly affected due to the weak

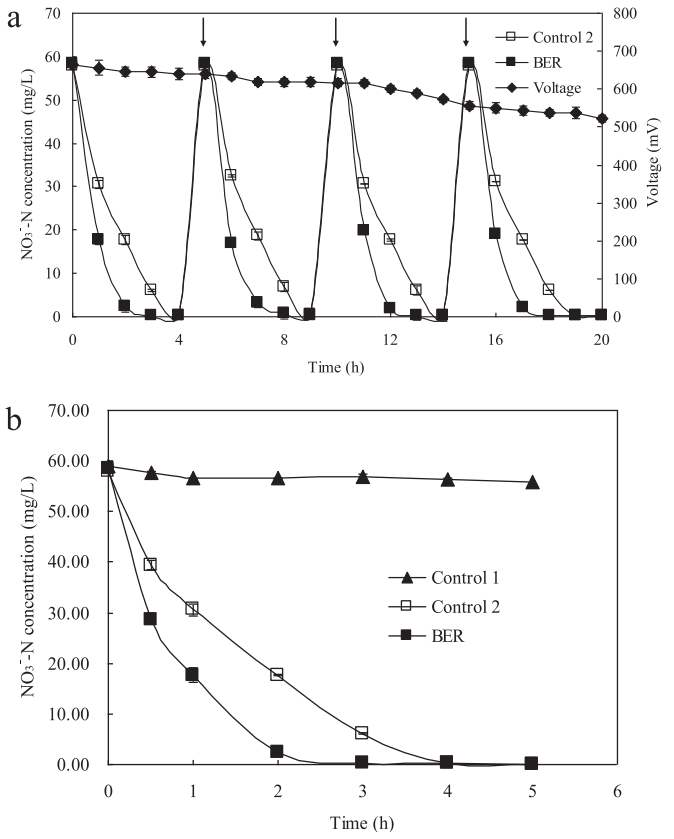

Fig. 3. NO_3^-–N removals in the BER and control sets with time (a) and in a typical operating cycle (b). A straight arrow indicated the replacement with fresh substrate.

current, thus these effects could not responsible for the significant enhancement of nitrate removal in present investigation [26]. Moreover, the anode and cathode potentials of BER during its operation were 200–412 mV and −409 to −215 mV (both vs. Ag/AgCl), respectively, suggesting the formation of electric field in the BER, which might function to bacteria directly, not to nitrate migration. Thus the observed improvement could be attributed to biological factors.

$$I_j = |Z_j| \cdot F \cdot C_j \cdot U_j \cdot X \cdot A \quad (1)$$

where I is current (A) of certain ion (NO_3^- or Na^+), Z is the charge numbers, F is Faraday constant (C mol^{-1}), C is ionic molar concentration (mol L^{-1}), U is the electric mobility (m^2 (s V)$^{-1}$), X is electric field intensity (V m^{-1}), A is the current cross-sectional area (m^2).

When a typical cycle finished, similar synthetic groundwater but without CH_3OH was added into the MFC intensified BER and nitrate concentration rarely changed during the new cycle, demonstrating that denitrifying bacteria were heterotrophic and cathode could not reduce nitrate by transferring electron directly to it, which again implied that the efficiency enhancement of nitrate removal with power from MFC were caused by improving the metabolism of denitrifying bacteria. After that, the same amount CH_3OH as Control 2 was added into the BER but the MFC was disconnected with it. Nitrate removal were also observed and its removal rate was slightly more quickly than that in the Control 2, both of them were lower than that in the MFC intensified BER, implying that the applied electric field acted on bacteria themselves and electrical stimulation from the MFC could improve the performance of BER in the aspect of nitrate pollution treatment. Electrons from MFC might be involved in the microbial electron transport chain during their respiratory metabolism and weak current could promote the bacteria proliferation, thus accelerating denitrification processes. The activity improvement and proliferation acceleration were further analyzed in Section 3.3.

Alternatively, the difference of the nitrate concentration between BER and Control 2 in Fig. 3a and b became larger and larger during the first 2 h of a typical operating cycle, indicating that bacterial nitrate removal was enhanced by the electrical connection of the MFC in the BER. Then this difference turned to insignificant until the end of the responding cycle due to low levels of the nitrate concentration in both systems. When the voltage outputs of the MFC decreased, the nitrate removal efficiency in the BER decreased accordingly, but certain extent improvement was also observed, compared with Control 2. This indicated that the electrical stimulation could widely exist in the range of voltage outputs of the MFC, and MFCs could be applied in the in-situ bioremediation of nitrate polluted groundwater for efficiency improvement. Further study would be conducted to examine the extent of electrical stimulation under different voltage outputs and different C/N ratios. In another aspect, the COD concentration in the BER decreased gradually with nitrate removal, nearly exhausted in the end of the operating cycle (from 58 ± 2 mg L^{-1} to 3 ± 0.5 mg L^{-1}), due to the consumption by bacteria in the reactor, thus avoiding extra organics discharge into groundwater environment. The COD concentration in the effluent of the Control 2 after the same operating cycle was slightly higher than that of the BER (about 5 ± 0.8 mg L^{-1}), again reflecting the enhancement of microbial metabolism by the applied electric field from the MFC in the BER.

3.2.2. Identification of reduction products

The reduction products of nitrate removal, both in the aqueous solution and gaseous phase of the BER were also monitored and investigated. There are mainly two pathways about nitrate related biological nitrogen cycle in nature, i.e. biological denitrification and dissimilatory nitrate reduction to ammonium (DNRA) [27]. Microbially mediated denitrification is an energy-yielding process catalysed by a range of intracellular enzymes to reduce one or both of the ionic nitrogen (N) oxides (NO_3^-, and nitrite NO_2^-) that may ultimately produce N_2 through the addition of two electrons per N-atom. The product of each reduction forms the substrate for the subsequent step in the process, as follows (Eqs. (2)–(5)) [19]:

$$NO_3^- + 2e^- + 2H^+ \rightarrow NO_2^- + H_2O \quad (2)$$

$$NO_2^- + 2e^- + 4H^+ \rightarrow NO + H_2O + 2OH^- \quad (3)$$

$$2NO + 2e^- + 2H^+ \rightarrow N_2O + H_2O \quad (4)$$

$$N_2O + 2e^- + 2H^+ \rightarrow N_2 + H_2O \quad (5)$$

while nitrate can also be biologically reduced to ammonium through DNRA process as Eq. (6).

$$NO_3^- + 8e^- + 10H^+ \rightarrow NH_4^+ + 3H_2O \quad (6)$$

Thus concentration variations of NO_2^-–N and NH_4^+–N in the aqueous solution with time in a typical cycle were measured and presented in Fig. 4a, where a relatively lower concentration of NO_2^-–N was detected, both in the BER and Control 2. NO_2^- is a primary intermediate product of denitrification, its existence indicated the denitrification process happened in these reactors. However, due to more toxicity to human beings, means should be taken to reduce nitrite generations, such as controlling the

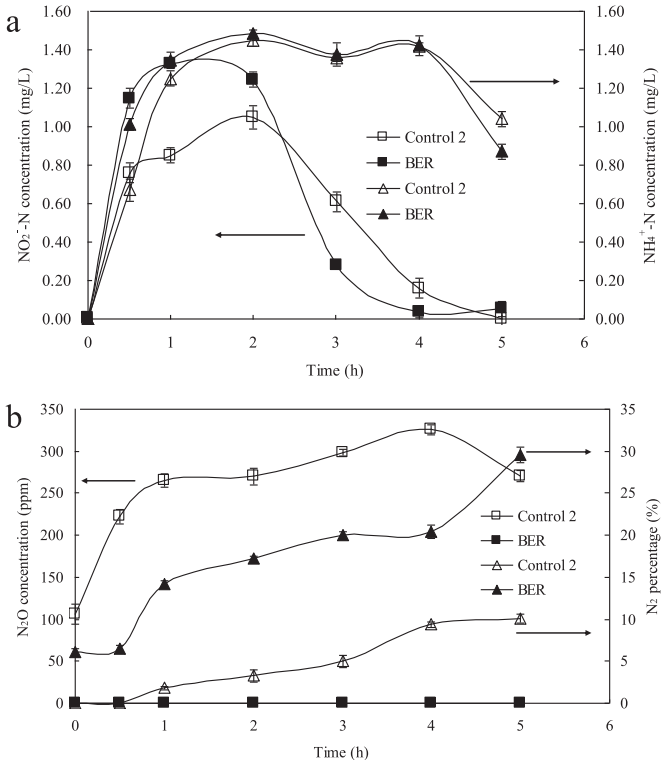

Fig. 4. Variations of reduction products both in the aqueous solution (a) and gaseous phase (b) of the BER and the control set during a typical operating cycle.

concentration of hydrogen at an appropriate value as nitrite reductase might be inhibited by its higher concentration in a traditional BER [28]. In the present study, NO_2^-—N consumption was accelerated by providing sufficient electrons from the MFC, thus its concentration was relatively lower than that in the Control 2. This meant electrochemical stimulation could enhanced the biological denitrification with reducing hazardous intermediate product accumulation. NH_4^+—N is the end products of DNRA process. It was also found in the aqueous solution and was presented in Fig. 4a, implying the DNRA process also occurred. Its concentration increased gradually both in the BER and the Control 2. DNRA was seen as a counterproductive process in denitrification studies and the balance between denitrification and DNRA depended on conditions of the reactor, in which, C/N value and carbon source species were important factors. NH_4^+—N concentration decreased along with the decrease of C/N [10], and DNRA was dominant when glycerin or glucose acted as electron donors [29]. In this study, CH_3OH acted as carbon source and the C/N level was not high, thus the NH_4^+—N concentration was kept relatively lower [30]. Moreover, a bit more NH_4^+—N was generated in the BER compared with that in the Control 2, indicating that electrical stimulation also accelerated the DNRA process.

In addition, the Anammox process is also an important biological process in nitrogen cycle which converts ammonium and nitrite to nitrogen gas. In present study, CH_3OH (5 mM) was the carbon source in the BER, which was the most potent inhibitor for Anammox process with its concentration as low as 0.5 mM, leading to complete and irreversible loss of activity of Anammox bacteria [31]. Thus the Anammox process was not taken into consideration.

N_2 is ultimately produced through a series of gaseous nitrogen oxide products (mainly NO and N_2O) which may escape to the atmosphere before being reduced to N_2 during biological denitrification process, thus concentrations of NO, N_2O and N_2 in the gas phase in a typical cycle were also monitored (Fig. 4b). NO is a recognised pollutant that contributes to stratospheric ozone destruction and radiative forcing in the troposphere [32]. Its concentration was very low and even not detected, both in the BER and Control 2, which indicated that NO was generated and then consumed right away, avoiding its escape and air contamination. N_2O is also produced as one of the obligatory intermediates during the denitrification process. It is an important greenhouse gas and can destruct the ozone layer [33], while denitrification completes the N-cycle by returning N_2 which is an environmentally benign gas to the atmosphere. Mitigation approaches should focus on ways to reduce the production of N_2O during denitrification and enhancing the reduction of N_2O to N_2 thus lowering the hazardous product release. Moreover, the reduction of N_2O to N_2 is a respiratory process in its own right, and incomplete denitrification often results in N_2O accumulation. In present study, N_2O was not observed in the BER, with abundant N_2 generation, elucidating that N_2O accumulation did not happen, due to the sufficient electron supplies from the MFC, which could reduce the generated N_2O (via reduction of NO) immediately and resulted in the observed sole accumulation of N_2. However, N_2O was accumulated with relatively higher concentration although N_2 was also observed, which had also been reported in the previous study [20]. While some studies revealed a nitrogen incorporation into gaseous nitrogen species (mainly N_2O) by exceeding the amount of available NO_2^-—N or NO_3^-—N [34,35]. This phenomenon also occurred in the Control 2 as concentrations of both NO_2^-—N and NO_3^-—N were higher than those in the BER. It is vital for the development of novel and effective N_2O mitigation technologies to reduce the production of N_2O during denitrification. This study implied that electrical stimulation from the MFC could proceed biological denitrification completely and reduced hazardous intermediates accumulation, exhibiting promising application prospects for enhancement of in-situ bioremediation for nitrate removal in groundwater.

3.3. Proliferation and activity enhancement of denitrification bacteria

Mircrobes responsible for nitrate removal in present study belongs two categories, ie. denitrification bacteria and DNRA bacteria. Their amounts and activities were evaluated and compared in the BER and Control 2.

3.3.1. Enumeration of microorganisms related to nitrate removal

As the contribution of electrons from the MFC to the total nitrate removals could be ignored, the enumeration of microorganisms related to nitrate removal was performed by MPN method with $C_6H_5Na_3O_7$ as sole electron donor. The positive/negative identification of MPN flasks for denitrification bacteria both in the BER and Control 2 were shown in Table 1, which showed that all four from 10^{-1} to 10^{-6} diluted MPN flasks were positive; three of the four 10^{-7} diluted samples were positive; three of the four 10^{-8} diluted samples were positive; only one of the four 10^{-9} diluted samples was positive; and all four 10^{-10} diluted samples were negative. Besides, results of diluted MPN samples of the Control 2 were also presented in Table 1 accordingly. According to the MPN count rule and our previous work [30], no matter how many repetitions, the quantitative indicator must be a three-digit number, and the first number must be all positive (4 in our study). If the following dilution was still growing, the number could be added to the third adjacent digit. Hence, Table 1 showed that the quantitative indicator of denitrification bacteria in the BER was '434', which was corresponded to 35.0 according to the MPN value table. It was multiplied by the dilution multiple of the first digit (the dilution multiple of 10^{-6} is 1,000,000), thus 3.5×10^7 of the MPN value in

Table 1
MPN counts for denitrification bacteria in the BER and Control 2.

Reactor		Dilution								
		10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}	10^{-10}
/	Replicates	4	4	4	4	4	4	4	4	4
BER	Positive	4	4	4	4	4	3	3	1	0
	MPN count	3.5×10^7 MPN/mL								
Control 2	Positive	4	4	4	4	3	3	1	0	0
	MPN count	3.5×10^6 MPN/mL								

the BER was obtained. The quantitative indicator of denitrification bacteria in the Control 2 was also '434', and the MPN value in the control 2 of 3.5×10^6 were obtained in the same way.

A mount of denitrification bacteria were found both in the BER and Control 2, responsible for high nitrate removal efficiencies, which was accordance with results got in our previous study [30], proving the reliability of present study. Moreover, the amount of denitrification bacteria in the BER was one order of magnitude larger than that in the Control 2, as bacteria proliferations can be promoted and contents of total protein in bacteria increase when proper DC current is applied [11]. Therefore, nitrate and intermediates produced through reduction process were removed much faster via electrical stimulation.

In addition, DNRA bacteria were also investigated based on the same method and results indicated that they also existed both in the BER and Control 2, but the number was very limited (only about 10^2), verifying the source of ammonia in the Section 3.2.2. The number of DNRA bacteria in the BER was also a few higher than that in Control 2, proving that electrical stimulation also worked on DNRA bacteria due to its no directionality.

3.3.2. Evaluation of microorganisms activities

Activities of denitrification bacteria and DNRA bacteria in the BER and Control 2 were also monitored. ATP content was chosen as an indicator to give information about microorganisms' activities in the present work. It is a molecule present in all viable cells, functioning as a carrier of chemical energy between catabolic reactions and all the cellular processes which require an energy input [21]. Assuming that it can be possible to use the concentration of ATP as a measure of viable cells of certain species, assays of ATP content had been widely used to characterize biomass viability [36]. The concentration variation of ATP in a typical cycle was shown in Fig. 5, which exhibited similar tendency. It could be found that ATP concentrations both in the BER and Control 2 started with high values, due to the addition of fresh organics and nutrients, after their almost exhaustions in the previous cycle. This implied that bacteria were activated which was favorable for following nitrate removal. It also illustrated that initial nitrate employed did not inhibited the bacteria activities. Subsequently, ATP concentrations decreased gradually due to the progressive consumptions of organics and nutrients. This tendency continued until the end of the operating cycle, with some minor fluctuations. Moreover, the ATP concentrations in the BER were much higher (about 1.5 folds) than those in the Control 2 during the operation, suggesting that bacteria were with higher activities in the former reactor. This could be contributed to the enhanced metabolism of bacteria with electrical stimulation. In one, cathodic reduction of either a mediator or part of the bacterial electron transport chain serves as the energy source for the bacteria. Sufficient electrons from the MFC could transfer to NADH first and nitrate finally through electron transport chain in bacteria cell. With electron transport accelerating, bacteria metabolism improved [37]. When electric field was applied, transmembrane potential occurred, which could promote the open of nutrients passages in the bacteria cytoplasmic membrane and increase its permeability. More nutrients flowed in with more cell contents such as enzymes and extracellular glycoprotein from bacteria metabolism out, thus exciting the increase of synthesis of ATP enzyme [37]. Additionally, both the applied electric field itself and reactive intermediates such as Fe^{3+}/Fe^{2+} and H could also improve the activities of ATP enzyme, therefore enhancing bacteria metabolism activities. In another aspect, there were as high as 27.18% of denitrifying bacteria existing in the inoculums [38], and these species would be dominant in the BER system after acclimatization [9], thus higher ATP concentration could be attributed to the activity enhancement of denitrifying bacteria for the acceleration of nitrate removal. Further study would be carried out with pure denitrifying bacteria under electrical stimulation in the following studies to eliminate influences of other microorganisms presented in the inoculums. In addition, considering the pollutant-removal characteristic of MFC, water connection of MFC and BER to further improve the pollutant removal efficiency as well as effects under different voltages with stacking MFCs connected in series and parallel will be conducted in the subsequent research.

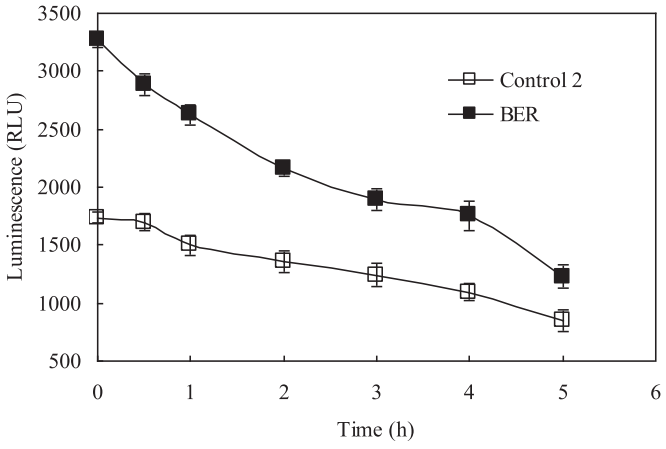

Fig. 5. Variations of ATP concentrations of the BER and the control set in a typical operating cycle.

4. Conclusions

Bacterial denitrification for nitrate removal in groundwater was enhanced with electrical stimulation from MFCs. When its voltage outputs in the range from 500 mV to 700 mV were applied directly to the BER, and the nitrate removal was accelerated, with less intermediates accumulation. 3.5×10^7 denitrification bacteria per milliliter liquid were detected, and the ATP concentrations also increased about 1.5 folds in the BER, implying that both proliferations and activities of denitrification bacteria were promoted. MFCs could work as a strengthening method to improve the efficiency of in-situ bioremediation of nitrate polluted groundwater.

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